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BclA and toxin antigens augment each other to protect NMRI mice from lethal *Bacillus anthracis* challenge

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Abstract

While proving highly effective in controlling Anthrax in farm animals all over the world currently attenuated live anthrax vaccines employed in a veterinary context suffer from drawbacks such as residual virulence, short term protection, variation in quality and, most importantly, lack of efficacy if administered simultaneously with antibiotics. These limitations have stimulated the development of non-living component vaccines which induce a broad spectrum immune response capable of targeting both toxemia (as in the case of PA based vaccines) and bacteraemia. To contribute to this several new approaches were tested in outbred NMRI mice for antibody titres and protectiveness. Plasmids encoding a recombinant toxin derived fusion peptide and a spore surface derived peptide were tested as DNA-vaccines in comparison to their protein counterparts utilising two adjuvant approaches and two DNA-vector backbones. The combination of two plasmids encoding

26 LFD1PAD4-mIPS1 and TPA-BclAD1D3-LAMP1, when delivered by GeneGun, protected 90% of
27 the animals against a lethal challenge with 25LD₅₀ spores of the Ames strain of *Bacillus anthracis*.
28 Single applications of either antigen component showed significantly lower protection rates,
29 indicating the beneficial interaction between anti-spore and anti-toxin components for an acellular
30 vaccine formulation.

31

32 **1. Introduction**

33 Anthrax is caused by *Bacillus anthracis*, a Gram-positive, spore forming, rod-shaped bacterium [1].
34 Spores gain access via cutaneous, oral or inhalational routes where they germinate and develop into
35 vegetative bacilli which then replicate and produce toxins which eventually kill the host [2]. The
36 pathogen expresses two major plasmid encoded virulence factors, a gamma-linked poly-D-glutamic
37 acid capsule (pX02 [3]) and a tripartite toxin (pX01 [4]) comprised of Protective Antigen (PA)¹,
38 Lethal Factor (LF)² and Edema Factor (EF)³ [5][6].
39 Current live attenuated veterinary anthrax vaccines are less than ideal. They can cause problems in
40 sensitive animals such as goats and llamas, protection is short term, variation in vaccine quality can
41 cause vaccine failure and most importantly the live nature of the vaccine prevents its efficacy if
42 delivered at the same time as antibiotics [7][8]. These limitations have stimulated the development
43 of non-living, component vaccines capable of inducing a broad spectrum immune response which
44 targets both toxemia and bacteraemia.
45 The strong correlation between toxin neutralising activity (tna)⁴ of PA-specific antibodies and
46 protection [9] has prompted efforts to develop vaccines based solely on domains which stimulate
47 antibodies with tna [10][11][12]. One such study which employed a fusion protein comprised of
48 domain 4 of PA (receptor binding site) and domain 1 of LF (PA binding site) protected mice against

1 PA – Protective Antigen

2 LF – Lethal Factor

3 EF – Edema Factor

4 tna – toxin neutralising activity

49 a subsequent lethal challenge with *B. anthracis* spores [13]. To further assess the immunogenic
50 value of this protein we administered it as a DNA-vaccine in two different vectors and compared its
51 activity to that seen against full length rPA83.

52 In addition to neutralising the action of toxins the spore can also be targeted to prevent the pathogen
53 from gaining a foothold in the infected individual [14][15]. Vaccination experiments with live
54 nonvirulent or formaldehyde-inactivated spores have shown that spore specific immune responses
55 can enhance the level of protection when given in combination with PA [16].

56

57 One such component is the *Bacillus* collagen like protein of *anthracis* (BclA)⁵ which forms hair-
58 like structures projecting from the spore surface and represents a major spore immunogen [17][18].
59 The removal of the collagen-like region (CLR⁶, domain 2) from BclA has no detrimental effect on
60 immunogenicity and results in a smaller peptide which is easier to incorporate into a
61 multicomponent vaccine [19][20]. In this study we determined the immunogenicity of a CLR-
62 deficient version of BclA called rBclAD1D3 when administered as a DNA-vaccine in two different
63 vectors.

64

65 For the DNA vaccine studies we employed two different plasmid backbones (pDNAVaccUltra and
66 NTC7382) which varied with regards to intracellular routing signals and immune stimulatory
67 elements [21]. To improve *in vivo* antigen presentation we utilised intracellular routing signals
68 which directed vaccine peptides to the MHC I and MHC II pathways. To target the MHC II
69 pathway [22] we employed tissue plasminogen activator (TPA)⁷ which routes newly expressed
70 proteins to the secretion pathway [23] and lysosome-associated membrane protein (LAMP1)⁸ which
71 directs proteins to the endosome [24][25]. To enhance MHC I presentation we employed ubiquitin

5 BclA – *Bacillus* collagen like protein of *anthracis*

6 CLR – collagen-like region

7 TPA – tissue plasminogen activator

8 LAMP1 – lysosome-associated membrane protein

72 which directs the associated protein to the proteasome [26][27].
73
74 To enhance the immunogenicity of the expressed proteins we investigated the utility of two
75 molecular adjuvants. Mouse interferon- β promoter stimulator 1 (mIPS-1)⁹ incorporated into the
76 backbone of the antigen encoding plasmid significantly induces type I interferon and interferon-
77 stimulated genes in a TLR-independent manner [28][29][30]. Mouse class II MHC trans-activator
78 (CIITA)¹⁰ up-regulates MHC expression [31][32] and was co-administered on a separate plasmid.
79 In comparison to the DNA vaccines, full length rPA and rBclA were tested as proteins alone and in
80 combination in the presence of a previously tested and approved lipopeptide adjuvant comprising
81 Pam3Cys-SKXXX, a TLR2/1 activator admixed with Pam₃Cys conjugated to the promiscuous T-
82 helper-cell epitope of the sperm whale myoglobin SFISEAIIHVLHSRHPG [33][34].
83 The overall aim of this study was to determine the ability of BclA to confer additional
84 protectiveness when given together with a toxin-specific vaccine.
85
86

87 **2. Material and Methods**

88 **2.1. Antigen preparation**

89 *E. coli* BL21-CodonPlus-RIL cells (Stratagene, La Jolla, CA) harboring the plasmid pREP 4
90 (Qiagen, Venlo, Netherlands) and pQE-30 (Qiagen) encoding either rPA83, rBclA or rLF were grown
91 and purified as described previously [35]. Proteins used for ELISA received no further treatment
92 while proteins used for vaccination were tested for endotoxin using the Limulus Amoebocyte Lysate
93 Endochrome-K test kit (Charles River, Wilmington, MA) as described by the manufacturer.
94 Endotoxin removal was performed via EndoTrap blue endotoxin removal system (Hyglos, Bernried,
95 Germany).

9 mIPS-1 – mouse interferon- β promoter stimulator 1

10 CIITA – mouse class II MHC trans-activator

96

97 **2.2. Preparation of DNA-vaccines**

98 Providing of vector-backbones (pDNAVaccUltra and NTC7382) including signal sequences,
99 cloning of respective gene sequences and purification of the constructs was undertaken by the
100 Nature Technology Corporation (Lincoln, NE). The sequence for murine CIITA (Mn01492) was
101 acquired from GeneCopoeia (Rockville, MD) and the sequence of mIPS-1 was used as given for
102 pUNO1-mIPS1 (Genebank: NM_144888.2). Antigens used comprised of rPA83 (2208 bp) [35],
103 LFD1PAD4 (1300 bp) [13] and BclAD1D3 (480 bp) [20]. Vaccines were prepared and applied as
104 described previously [35].

105

106 **2.3. Administration of vaccines and challenge**

107 Trials were performed using 8-12 weeks old female outbred NMRI mice (Charles River).
108 Immunisation groups comprised of 10 animals, while groups which only received an adjuvant
109 (lipopeptide or CIITA) contained 5 individuals.

110 Mice were immunised 3 times at intervals of 2 weeks. Challenge was performed 3 weeks after the
111 last immunisation and mice were monitored for survival for up to 4 weeks. Blood was taken prior to
112 immunisation, before challenge and after the end of the challenge. All data presented is referring to
113 sera taken before the challenge unless stated otherwise. Preparation, vaccination and challenge of
114 the mice was performed under anesthesia with Isofluran (Actavis, Weiterstadt, Germany) using a
115 ventilated Box.

116 Mice immunised with protein received a 200 µl dose s.c. in the neck containing 25 µg of each
117 antigen and 50 µg of the lipopeptide adjuvant (EMC microcollections, Tuebingen Germany) diluted
118 in sterile endotoxin free PBS (Sigma-Aldrich, St. Louis, MO). Mice immunised with DNA-vaccines
119 were shaved 1-2 days before the immunisation. Each mouse received 2 cartridges containing a total
120 of 3 µg of DNA (6 µg for the combination) per immunisation applied via GeneGun (tab 1). For the

challenge, a dose of 200 µl of a fully virulent Ames strain containing ~1000 spores (25LD₅₀) for all DNA-vaccines and ~2000 spores (50LD₅₀) for all protein vaccines was administered s.c. in the neck. The increased challenge dose for the protein vaccines was utilised due to the residual protectiveness of the lipopeptide alone observed when challenged with lower spore doses (data not shown), thus normalising its effect to allow for a better discrimination between different antigens and applications. Mice that died during the challenge or were killed via CO₂ after the end of the challenge were dissected to prepare liver and spleen which were then plated on blood agar to verify the presence of the pathogen.

129

130 **2.4. Serology**

ELISA was performed as described previously [35]. Secondary antibodies comprised of horseradish peroxidase conjugated polyclonal goat anti-mouse IgG (Sigma-Aldrich), IgG1 and IgG2a (Acris, San Diego, CA). Endpoint titres were defined as the reciprocal of the highest serum dilution that resulted in an absorbance greater than two standard deviations above the average of the negative control serum sample (pooled naive sera of the according group) with a minimum OD_{414nm} value of 0.1.

Sera were analysed for neutralising antibody titres via in vitro toxin neutralising assay (TNA) as published previously [35]. The neutralisation titre (NT) corresponds to the inverse serum dilution at which the toxin neutralisation yielded 50% (NT₅₀). The NT₅₀ was estimated using the SigmaPlot regression wizard (4-parameter sigmoid regression curve). Detailed Titres are only described for sera taken before the challenge.

142

143 **2.5. Statistics**

Estimated antibody titres of different groups were compared via Mann-Whitney U-test. Survival rates were analysed through log rank test, taking full days survived into consideration. Correlations

146 between titres and survival were estimated via spearman-rank correlation test. For all
147 statistical purposes a P-value of 0.05 and smaller was considered significant, resulting in a critical
148 value of 0.564 for r_s for quantities of 10.

149

150 **3. Results and discussion**

151 **Addition of rBclA to rPA83 increased the level of protection when applied together as proteins**

152 Groups of mice vaccinated with either rPA83, rBclA or a combination of both together with
153 lipopeptide adjuvant induced significant IgG antibody titres with a strong IgG1 emphasis against
154 their respective antigens (fig. 1 and 2). The measured antibody titres as well as the NT₅₀-titres
155 (fig. 3) for the groups receiving either rPA83 or rBclA alone were similar to or higher than those
156 seen in the group given both proteins suggesting no synergistic effects or shift in subclass
157 dominance.

158 Each antigen when given alone did not provide significant protection while the combination
159 achieved 70% protection (tab. 1; fig. 4A) indicating that rBclA and rPA83 augment each others
160 ability to protect. These findings are similar to those reported in a previous study where A/J mice
161 were challenged with spores of the Sterne strain of *B. anthracis* [14]. The authors of this study
162 concluded that BclA enhanced protection by inducing the opsonization of spores and stimulating the
163 production of antibodies which also inhibited germination. In addition PA-specific antibodies have
164 been shown to promote spore opsonization and the inhibition of germination in a similar manner to
165 BclA [36]. Thus the protection seen in animals given rPA83 and rBclA may be the result of a
166 synergy in the anti-spore effect rather than a feature of anti-toxin properties. This would explain
167 why we saw no correlation between survival and antibody titres ($r_s \leq \pm 0.53$). This applied also to
168 the NT₅₀-titres of animals immunised with rPA83 as they had generated high toxin neutralising
169 antibodies, a feature which has previously been shown to correlate with protection in other animal
170 models [9][37][38][39]. However to our knowledge such a correlation has not been recorded for

171 outbred mice challenged with Ames.

172 Comparison of the IgG subclass spectrum revealed a strong Th₂ bias, with IgG1 titres equating IgG
173 titres strongly ($r_s \geq +0.81$). Nonetheless significant, but highly scattered IgG2a antibody titres
174 against both antigens were raised in the majority of the animals.

175

176 **DNA-vectors encoding for toxin components were able to significantly protect outbred NMRI**
177 **mice from lethal challenge**

178 In a previous study we reported that the fusion protein LFD1PAD4 was able to fully protect A/J
179 mice against i.p. challenge with $\sim 10^5$ spores of the Sterne vaccine strain [13]. To determine if a
180 similar level of protection could be achieved when the antigen was presented in the context of a
181 DNA vaccine we introduced the gene encoding the fusion protein into two different DNA vectors
182 and compared the resulting immune responses to those seen using a DNA vaccine expressing full
183 length rPA83. The latter had been shown to significantly protect A/J mice from a lethal challenge
184 with the attenuated STI-1 vaccine strain [35] but not outbred mice from challenge with spores of the
185 fully virulent Ames strain [40].

186 The DNA-vaccines elicited significantly lower (1-2 log₁₀) anti-PA-antibody and NT₅₀-titres
187 (fig. 1 and 3) than the protein vaccines which contained rPA83 (tab 1). While the TPA-LFD1PAD4-
188 mIPS1 and TPA-rPA83-LAMP1 constructs stimulated similar anti-rPA83-IgG and -IgG1 titres they
189 were significantly higher than those seen using TPA-LFD1PAD4-LAMP1 which failed to elicit anti-
190 rPA83 titres in the majority of the animals and thus was not distinctive of the pre-immune sera.

191 Of the two DNA constructs which contained LF the TPA-LFD1PAD4-mIPS1 vector induced higher
192 anti-rLF-IgG and -IgG1 titres (fig. 5) and for both constructs the LF specific antibody response
193 before the challenge exceeded that seen against PA as has been noted previously [13]. A positive
194 correlation between anti-rPA83- and anti-rLF-IgG titres to NT₅₀-titres ($r_s \geq +0.66$) was seen for
195 TPA-LFD1PAD4-mIPS1 suggesting that the fusion protein stimulated the production of toxin

196 neutralising antibodies. Indeed significant NT₅₀-titres were only raised in individuals immunised
197 with TPA-LFD1PAD4-mIPS1. With regards to the IgG subclass balance all of the DNA constructs
198 failed to stimulate an IgG2a response for rPA83 and rLF indicating a strong Th₂-specific immune
199 response often seen with GeneGun applications [40][41][41]
200 When challenged with 25LD₅₀ of fully virulent Ames strain spores a level of protection of between
201 30-40% was accomplished (tab. 1). Interestingly, only the surviving individuals immunised with
202 TPA-LFD1PAD4-LAMP showed a measurable anti-rPA83-IgG titre before the challenge whereas
203 the individuals that succumbed to infection did not, resulting in a strong correlation of anti-rPA83-
204 IgG titres with survival ($r_s = +0.888$). Furthermore, anti-rLF-IgG titres were induced in all animals
205 and showed a correlation to survival ($r_s = +0.685$) and anti-rPA83-IgG titres ($r_s = +0.885$). A notable
206 difference concerning the subclass bias of the two adjuvants tested (CIITA and mIPS-1) was not
207 present since their mode of action results in an up-regulation of the MHC-genes neither favouring
208 IgG1 nor IgG2a responses. However the variable immunogenicity of the different vectors might be
209 attributed to the different adjuvants utilised. In conclusion the toxin vectors examined in this study
210 elicited slightly lower PA-specific antibody titres than seen in an earlier study, but were equally
211 protective [40].

212

213 **DNA-vectors encoding for BclAD1D3 possibly stimulated sterile immunity and protected 50%** 214 **of the animals**

215 BclAD1D3 was cloned into two different DNA vaccine vectors, one in which the gene was flanked
216 by TPA and LAMP1 and a second in which BclAD1D3 was directed to the proteasome by the
217 incorporation of an N-terminal ubiquitin signal sequence. Both constructs elicited robust anti-
218 rBclA-IgG, -IgG1 and -IgG2a antibody responses (fig. 2) comparable to those seen for full length
219 rBclA given as a protein. We saw no significant difference in the magnitude of the immune response
220 for either DNA vaccine suggesting that the various routing signals had no detectable influence. The

only discernable difference between both concerned the IgG2a titres, which were detectable in all individuals immunised with TPA-BclAD1D3-LAMP1 but not when BclAD1D3-Ubiquitin was applied. It is possible that by using a GeneGun to deliver each DNA vaccine any beneficial effect provided by targeting routing was masked [41][42]. Our previous study with full length BclA applied via GeneGun in a vector containing a different secretion signal, depicted a similar antibody spectrum with a distinctive IgG1 bias [40].

Both of the vaccines tested in our study protected 50% of the animals when challenged with fully virulent spores (fig. 1B). Analysis of the blood of animals which have survived challenge gave the indication of a possible sterile infection as inferred from lack of PA antibodies [43]. To our knowledge the level of protection achieved in this study following immunisation with BclAD1D3 exceeds that previously reported for BclA in protein or DNA vaccine form [14][40][44][20].

A combined DNA vaccine comprising TPA-LFD1PAD4-mIPS1 and TPA-BclAD1D3-LAMP1 stimulated significant protection against a lethal spore challenge

To determine if we could further increase the level of protective immunity we co-immunised mice with TPA-LFD1PAD4-mIPS1 and TPA-BclAD1D3-LAMP1 and subjected them to a lethal spore challenge. The TPA-BclAD1D3-LAMP vector was selected for these studies as it had previously been reported that DNA-vectors containing ubiquitin reacted adversely in the presence of other DNA vector signal sequences [45]. Following a lethal spore challenge (25LD₅₀) 90% of the animals survived and there was an increase in time to death for the single animal that succumbed to infection (tab 1).

This increased protection was mirrored by an increase in the magnitude of the anti-rPA83-IgG and -IgG1 titres (fig. 1) in that they significantly exceeded those of the single vectors although they were still significantly lower than those seen in animals who had received protein vaccines containing rPA83. The enhanced antibody titres against rPA83 were not associated with a higher NT₅₀-titre

246 suggesting that the immune response was directed against other epitopes, possibly targets that
247 promote opsonization or inhibit germination. While the boost in anti-rPA83 titres as a consequence
248 of co-administration of spore antigens has been seen previously, it was in animals who had been
249 immunised with a protein formulation [44]. Our corresponding protein combination did not mimic
250 this trend.

251 The anti-rLF-IgG titres observed following co-administration were on the whole similar to those
252 seen after immunisation with TPA-LFD1PAD4-mIPS-1 alone and showed less scattering. The NT₅₀-
253 titre correlated strongly with anti-rLF-IgG ($r_s = +0.85$) and -IgG1 titres ($r_s = +0.918$) and weakly
254 with anti-rPA83 IgG and -IgG1 titres ($r_s \geq +0.61$). This was also true when TPA-LFD1PAD4-mIPS1
255 was given alone although the correlations were much weaker, thus the tna was positively influenced
256 for both parts of the antigen alike when combined with BclA under the presence of both adjuvants
257 while the overall titres generated were diversified.

258 Surprisingly the anti-rBclA titre of the DNA-vaccine combination and to a lesser extend that of the
259 protein combination, was significantly lower than that seen for either single vaccine application
260 (fig. 2). While an antagonistic effect of BclA on PA titre has been observed in other studies and has
261 been ascribed in part to “antigenic competition” [14][46][44] a negative effect of PA on BclA titres,
262 as seen here, has not been reported. Apart from the possibility of DNA-vector interferences [45] and
263 the possible influence of the presence of the adjuvant mIPS-1 in addition to CIITA we have no
264 explanation for this phenomenon.

265 However, the overall individual titres measured for the DNA-combination showed less scattering
266 within the group compared to the titres seen in the groups given a single vector. Thus BclA and PA
267 given together might have a balancing effect leading to a more stable immune response which
268 might contribute to enhanced survival.

269 In conclusion a DNA vaccine comprising a combination of plasmids encoding spore and toxin
270 targets conferred significantly greater protection than that seen for the individual DNA plasmids

271 ($P \leq 0.042$), the single protein components ($P \leq 0.00008$) and the vectors tested in our previous
272 study [40]. Furthermore it was found to be as effective as a comparable protein combination
273 ($P = 0.251$). To our knowledge this is the first example of a vaccination strategy against Anthrax in
274 which the protective immunity achieved using a DNA based approach exceeded that seen with
275 protein equivalents [40].

276

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284

285

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Table 1

Groups	Antibody titres ^d				Vaccine efficacy (%)	survivors / total	Mean time to death ^g
	PA IgG	LF IgG	BclA IgG	TNA			
<u>Protein vaccination</u>							
Lipopeptide adjuvant	<200	n.d.	<200	<100	0% ^e	(0/5) ^e	4.2 ± 3.3
Lipopeptide + rBclA	<200	n.d.	257066 ± 149351	<100	10% ^e	(1/10) ^e	3.4 ± 0.7
Lipopeptide + rPA83	561803 ± 113102	n.d.	<200	14564 ± 4543	10% ^e	(1/10) ^e	3.7 ± 1.4
Lipopeptide + rBclA + rPA83	415030 ± 199480	n.d.	123442 ± 42637	15581 ± 9683	70% ^e	(7/10) ^e	5.2 ± 3.2
<u>DNA vaccination</u>							
CIITA ^a	<200	<200	<200	<100	0% ^f	(0/5) ^f	3.1 ± 0.7
TPA-LFD1PAD4-mIPS1 ^a	22488 ± 28511	80682 ± 93200	<200	691 ± 772	30% ^f	(3/10) ^f	4.1 ± 1.2
CIITA + TPA-LFD1PAD4-LAMP1 ^b	2135 ± 4065	14443 ± 9708	<200	<100	40% ^f	(4/10) ^f	4.2 ± 0.7
CIITA + TPA-PA83-LAMP1 ^b	31645 ± 26067	<200	<200	239 ± 524	30% ^f	(3/10) ^f	4.0 ± 1.0
CIITA + TPA-BclAD1D3-LAMP1 ^b	<200	<200	166381 ± 106094	<100	50% ^f	(5/10) ^f	4.2 ± 1.6
CIITA + BclAD1D3-Ubiquitin ^b	<200	<200	221171 ± 150564	<100	50% ^f	(5/10) ^f	4.5 ± 1.7
CIITA + TPA-LFD1PAD4-mIPS1 + TPA-BclAD1D3-LAMP1 ^c	60015 ± 32158	57728 ± 37181	34528 ± 15256	463 ± 682	90% ^f	(9/10) ^f	9.0 ± 0.0

^a immunisation with uniform bullets, resulting in a dose of 3 µg per vector per immunisation

^b immunisation with mixed bullets (1:1) reducing the effectively applied amount of each vaccine vector to 1.5 µg per vaccination

^c immunisation with mixed bullets (1:2:1) reducing the effectively applied amount of each vaccine vector to 1.5, 3 and 1.5 µg respectively per vaccination

^d group mean IgG or NT₅₀ titres ± standard deviation before the challenge

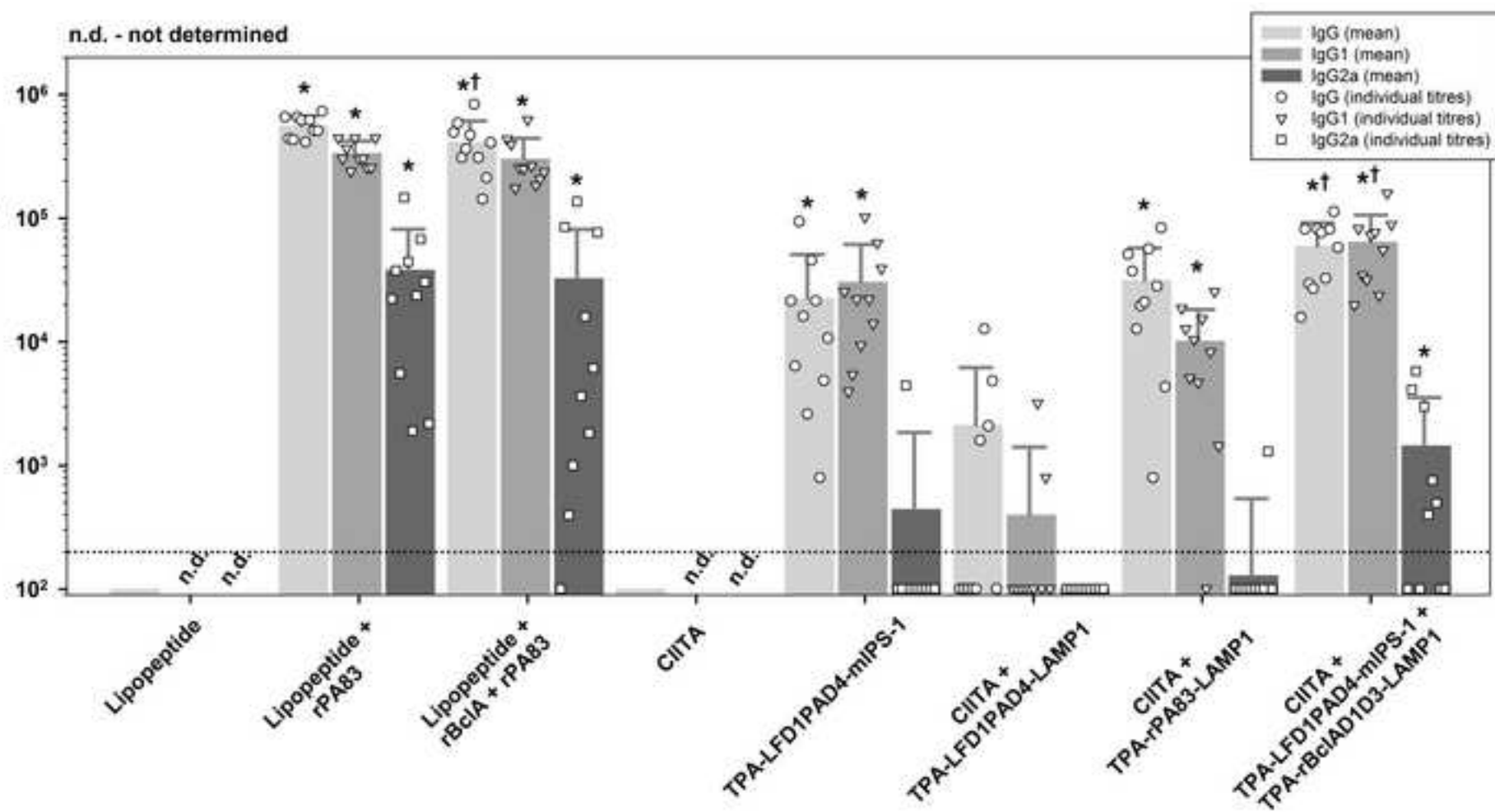
^e protein groups were challenged with 50LD₅₀

^f DNA groups were challenged with 25LD₅₀

^g time to death in days post challenge ± standard deviation

1

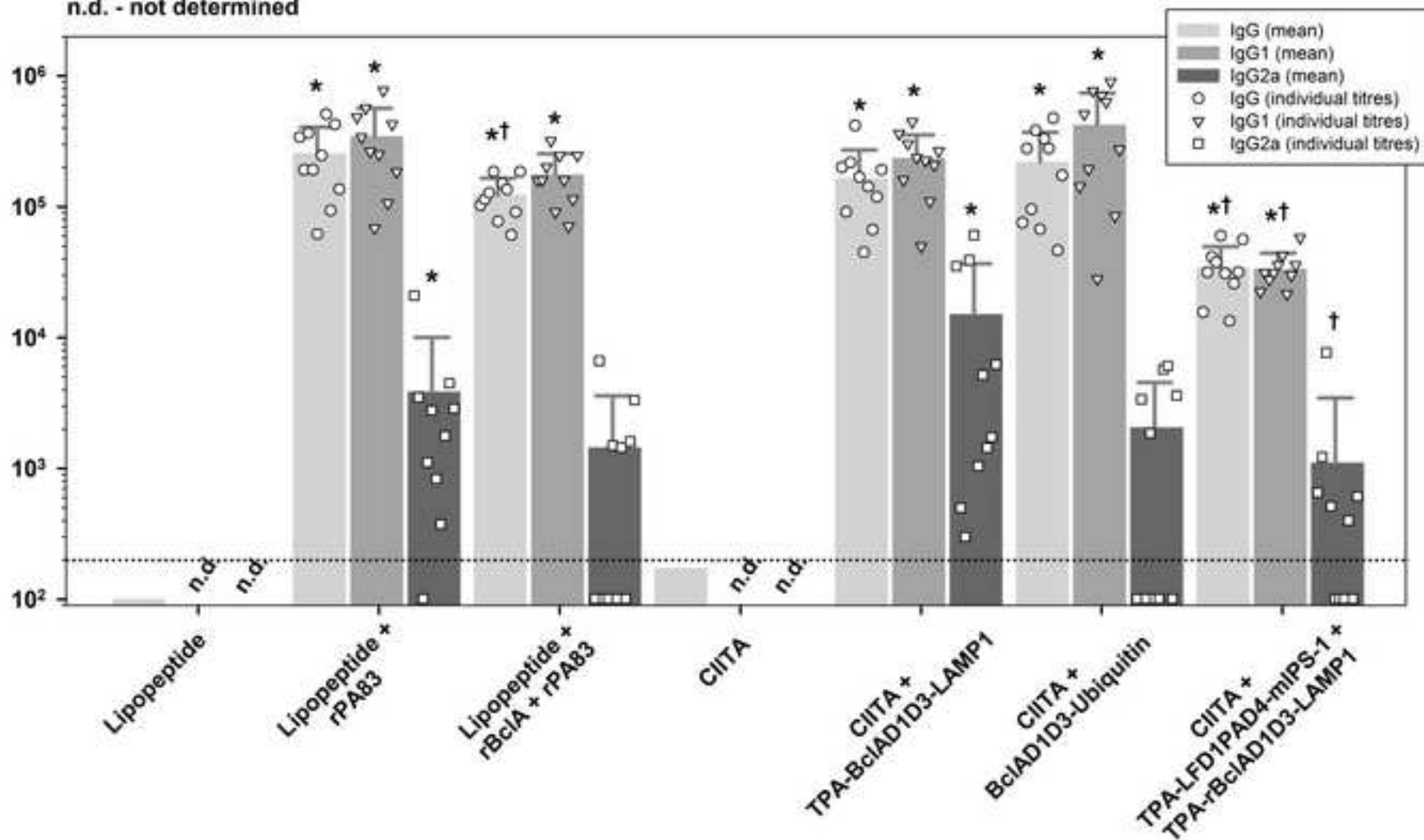
Anti-rPA83 antibody titre



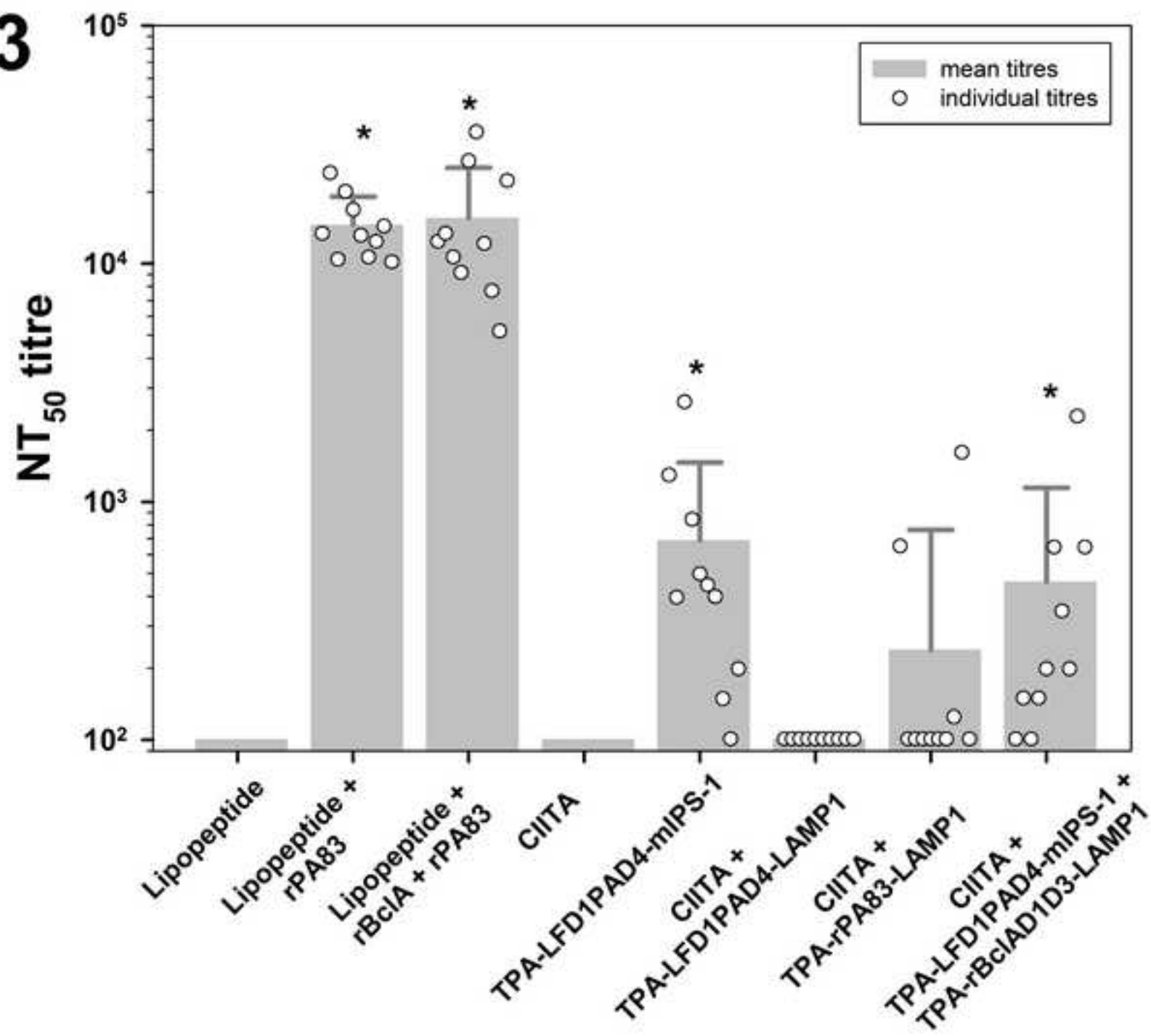
2

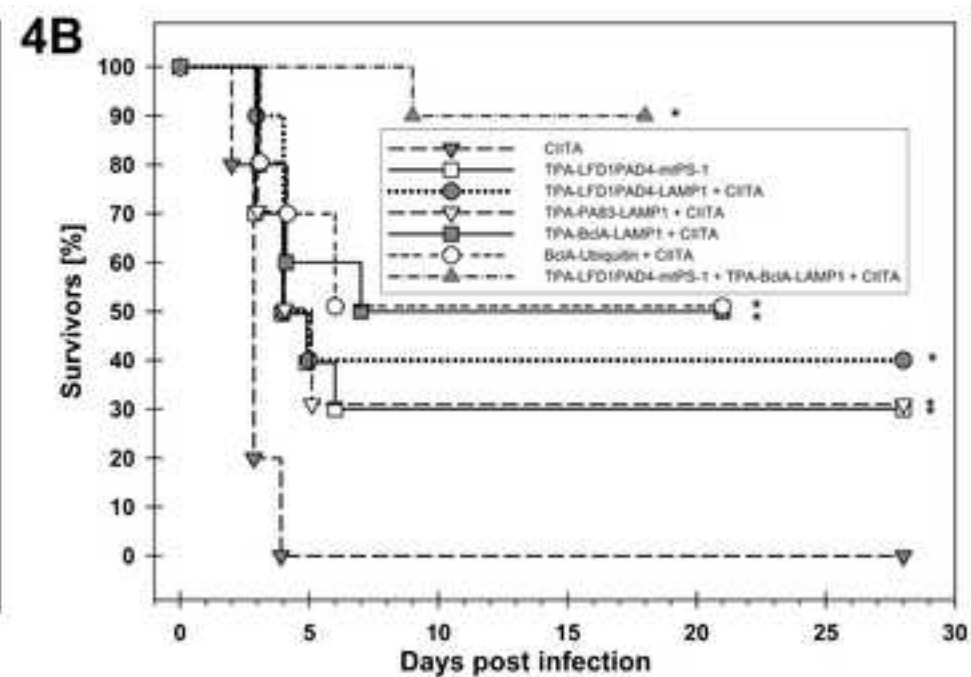
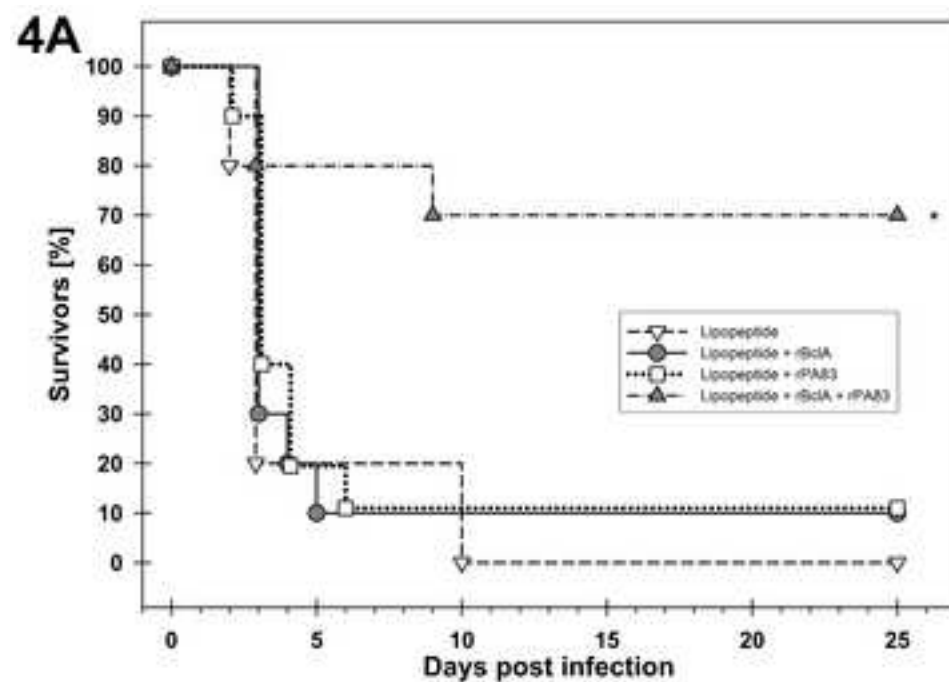
Anti-rBclA antibody titre

n.d. - not determined



3





5

Anti-rLF antibody titre

